The rulB gene of plasmid pWW0 is a hotspot for the site-specific insertion of integron-like elements found in the chromosomes of environmental *Pseudomonas fluorescens* group bacteria.

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Summary

The rulAB operon of *Pseudomonas* spp. confers fitness traits on the host and has been suggested to be a hotspot for insertion of mobile elements that carry avirulence genes. Here, for the first time, we show that rulB on plasmid pWW0 is a hotspot for the active site-specific integration of related integron-like elements (ILEs) found in six environmental pseudomonads (strains FH1–FH6). Integration into rulB on pWW0 occurred at position 6488 generating a 3 bp direct repeat. ILEs from FH1 and FH5 were 9403 bp in length and contained eight open reading frames (ORFs), while the ILE from FH4 was 16 233 bp in length and contained 16 ORFs. In all three ILEs, the first 5.1 kb (containing ORFs 1–4) were structurally conserved and contained three predicted site-specific recombinases/integrases and a tetR homologue. Downstream of these resided ORFs of the ‘variable side’ with structural and sequence similarity to those encoding survival traits on the fitness enhancing plasmid pGRT1 (ILEFH1 and ILEFH5) and the NR-II virulence region of genomic island PAGI-5 (ILEFH4). Collectively, these ILEs share features with the previously described type III protein secretion system effector ILEs and are considered important to host survival and transfer of fitness enhancing and (a)virulence genes between bacteria.

Introduction

Bacteria within the genus *Pseudomonas* are found in a wide range of terrestrial and aquatic natural and clinical environments and demonstrate remarkable metabolic and physiological versatility including the potential for pathogenicity (Morris *et al.*, 2000; 2007; 2008; Riffaud and Morris, 2002). This has been particularly illustrated by sequenced genomes (Rodríguez-Palenzuela *et al.*, 2010; Ortel *et al.*, 2011; Ramírez-Díaz *et al.*, 2011; Winsor *et al.*, 2011; Yu *et al.*, 2011; Patel *et al.*, 2012). These have revealed the extent of the horizontal transfer of mobile genetic elements such as phage, transposons and insertion sequences, and genomic and pathogenicity islands (Roy *et al.*, 2010; Martinez *et al.*, 2012; Morales-Espinosa *et al.*, 2012; Tang *et al.*, 2012; Wu *et al.*, 2012), and the mosaic nature of bacterial genomes in general (Hall, 2012; Marttinen *et al.*, 2012).

The rulAB operon in *Pseudomonas* spp. has been shown to confer fitness traits including ultraviolet (UV) tolerance on its host (Sundin *et al.*, 1996; Gibbon *et al.*, 1999) and to be involved in the SOS response and the growth advantage in stationary phase phenotype (Tark *et al.*, 2005; Kivisaar, 2010). The operon is common to both the chromosomes and plasmids of pseudomonads (Sundin *et al.*, 2000; Zhao *et al.*, 2005; Cazorla *et al.*, 2008). In the latter, it is usually located close to transfer or mating pair formation encoding regions in the core.
backbone, ensuring that it is one of the first regions transferred during conjugation (Gibbon et al., 1999).

Analysis of Pseudomonas genotypes demonstrated that rulAB is common in an intact or an interrupted form. Its function and benefit to bacterial hosts is still relatively poorly understood (Jackson et al., 2011). Arnold and colleagues (2001) found that the avirulence gene avrPpiA resided in a 4.3 kb region that interrupted the rulB gene in P. syringae pv. pisi and concluded that the rulB gene may be a hotspot for insertion of mobile regions of DNA. Interruption of the rulB gene by integration of integron-like elements (ILEs) led to the postulation that the rulAB promoter controls the expression of integrase under the regulation of LexA repressor protein (a LexA binding site can be found upstream of rulAB) (Jackson et al., 2011). This association is broad, with similar disruptions of rulAB-related DNA repair genes rumAB, umuDC, impAB, mucAB, samAB and ruvAB in a range of bacteria including the insertion of the SXT conjugative element that confers pathogenicity and is embedded in rumB of Vibrio cholerae (Hochhut et al., 2001).

The 117 kb plasmid pWW0 is the archetypal plasmid of the IncP-9, a family of large self-transmissible plasmids found mainly in pseudomonads that harbour genes for antibiotic and heavy metal resistance and the biodegradation of mono-aromatic and polycyclic compounds (toluene/xylene and naphthalene) (see Sevastsyanovich et al., 2008). In pWW0, these genes are harboured within the 70 kb transposon Tn4653, with the remainder of the plasmid containing the core backbone functions. Although classified as a narrow host range plasmid, pWW0 can transfer at frequencies as high as $10^{1\text{ to } 1}$ to 1 transconjugant per recipient cell between pseudomonads (Nakazawa, 1978; Ramos et al., 1987) and can transfer to enterobacteriaceae at lower frequencies (see Ramos et al., 1997). It also has the capability for retrotransfer (Ronchel et al., 2000). Carriage of pWW0 has been shown to be beneficial to host bacteria not only through traits encoded by the accessory genes within Tn4653 but also from those encoded by the rulAB-homologue genes (termed ruvAB; Greated et al., 2002) within the core backbone. In pWW0, these genes are located between positions 5405–7034 and have been shown to encode a DNA polymerase Pol V homologue that significantly increases the evolutionary fitness of the P. putida host bacteria during prolonged nutritional starvation (Tark et al., 2005).

In the present study, we report for the first time the active integration of a group of related ILEs from environmental Pseudomonas spp. isolates into plasmid pWW0 and show that insertion into rulAB operon and its homologues in other genera is potentially of key importance to the adaptation and survival of these bacteria.

### Results

#### Discovery of a novel ILE

During an investigation of plasmid-encoded copper resistance in environmental pseudomonads recovered in a previous study (Pickup, 1989), we attempted to cure native plasmids from these strains by incompatibility using the IncP-9 toluene-degrading plasmid pWW0. After conjugation between P. putida PaW340 (pWW0) and environmental isolate FH1 (Table 1), and subsequent verification of FH1 (pWW0) transconjugants by restriction digest analysis of pWW0FH1, we observed that plasmid pWW0 had acquired an extra region of DNA and that this process was repeatable. Restriction mapping showed the insert to be around 10 kb in size; the region was subsequently cloned on a PsiI fragment into vector pBR325, and the recombinant plasmid designated pFBA1001 (not shown). This region was subsequently shown by DNA hybridization against genomic DNA from plasmid-cured FH1 to be chromosomally located (not shown).

The 10 kb region of pWW0FH1 in pFBA1001 was sequenced, and a complete assembly was constructed. Putative open reading frames (ORFs) were identified, and the DNA and protein sequences within this region were aligned with sequences in the databases. The PsiI fragment was 10 165 bp in length and was flanked on either side by 480 and 282 bp of a disrupted rulB gene. The rulB-flanked region was therefore 9403 bp in length and contained eight ORFs (Table 2). Alignments revealed that all eight ORFs had the closest nucleotide and protein identity with ORFs 26–35 in plasmid pGRT1 of P. putida DOT-T1E that is tolerant to high concentrations of toluene via efflux pumping (Molina et al., 2011) (Table 2). Notably, ORFs 1–3 were phage integrases/site-specific recombinases. The predicted protein of ORF1 possessed the C-terminal R-H-R-Y motif of tyrosine recombinases and multidomains of XerC and XerD recombinases, and was therefore designated xerD (Supporting information Fig. S1). ORF2 and ORF3 were also putative site-specific recombinases that possessed the INT_REC_C conserved domain (not shown).

The only significant difference between the pFBA1001 element and its counterpart region on pGRT1 was the presence in pGRT1 of an IS4-like transposase (ORF29) that is absent from pFBA1001. In pGRT1, this transposase divides ORF28 and ORF30 (also both predicted to encode site-specific recombinases), and its in silico deletion from pGRT1 results in the same sequence found in ORF3 (int/rec) on pFBA1001, suggesting the possibility of an insertion event (not shown). As in pFBA1001, ORFs 26–35 in pGRT1 are flanked by ruvAB (rulAB) genes (ORFs 25 and 36) homologous with rulAB of pWW0. In addition, the region is oriented in the same way as in pFBA1001.
The sequence of the ORF5 predicted protein shares 96% identity with that encoded by ORF32 on pGRT1 and was predicted to be an SdiA-regulated motif protein involved in modulation of the TtgGHI efflux pump (Molina et al., 2011). Similarly, ORF7 that shares 96% protein sequence identity with pGRT1 ORF34 was predicted to encode a universal stress response protein UspA, which in the latter conferred a two-order of magnitude survival advantage to toluene shock after moderate exposure to toluene stress (Molina et al., 2011). ORF8 was homologous to ORF35 on pGRT1 and was predicted to encode a sulphate permease that has been shown to be involved in siderophore production (possibly via the release of a pseudobactin-like siderophore (see Molina et al., 2011)). Collectively, the presence of a xerD integrase, tetR gene and other possible fitness-enhancing traits in the mobile region from FH1 were suggestive of an integron-like structure. For this reason, the FH1 element was designated an ILE.

### Table 1. Bacterial strains and plasmids.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Environmental pseudomonads</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FH1 (isolated in 1985)</td>
<td>Chromosomally located ILEFH1; Km(^{\text{r}}), Sm(^{\text{s}})</td>
<td>This study</td>
</tr>
<tr>
<td>FH2 (isolated in 1995)</td>
<td>Chromosomally located ILEFH2; Km(^{\text{r}}), Sm(^{\text{s}})</td>
<td>This study</td>
</tr>
<tr>
<td>FH3 (isolated in 1995)</td>
<td>Chromosomally located ILEFH3; Km(^{\text{r}}), Sm(^{\text{s}})</td>
<td>This study</td>
</tr>
<tr>
<td>FH4 (isolated in 1995)</td>
<td>Chromosomally located ILEFH4; Km(^{\text{r}}), Sm(^{\text{s}})</td>
<td>This study</td>
</tr>
<tr>
<td>FH5 (isolated in 1995)</td>
<td>Chromosomally located ILEFH5; Km(^{\text{r}}), Sm(^{\text{s}})</td>
<td>This study</td>
</tr>
<tr>
<td>FH6 (isolated in 1995)</td>
<td>Chromosomally located ILEFH6; Km(^{\text{r}}), Sm(^{\text{s}})</td>
<td>This study</td>
</tr>
<tr>
<td>Control strains/constrasts</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas putida</em> PaW340</td>
<td>Sm(^{\text{r}}); trp−</td>
<td>DSM 2112</td>
</tr>
<tr>
<td><em>P. putida</em> PaW340 (pWWW0)</td>
<td>Sm(^{\text{r}}); TOL; trp−</td>
<td>Franklin and Williams (1980)</td>
</tr>
<tr>
<td><em>P. putida</em> EEZ15 (pWWW0::Km(^{\text{r}}))</td>
<td>Sm(^{\text{r}}); Km(^{\text{r}})</td>
<td>Ramos-Gonzalez and colleagues (1994)</td>
</tr>
<tr>
<td><em>P. putida</em> PaW340 (pWWW0::Km(^{\text{r}}))</td>
<td>Sm(^{\text{r}}); TOL; Km(^{\text{r}})</td>
<td>This study</td>
</tr>
<tr>
<td><em>P. putida</em> PaW85 (pWWW0::rulAB::Km(^{\text{r}}))</td>
<td>Sm(^{\text{r}}); TOL; Km(^{\text{r}})</td>
<td>Tark and colleagues (2005)</td>
</tr>
<tr>
<td><em>Escherichia coli</em> HB101 (pFBA1001)</td>
<td>PstI fragment containing ILEFH1, and truncated rulAB</td>
<td>This study</td>
</tr>
<tr>
<td><em>P. putida</em> PaW340 (pWWW0::KmR::ILEFH1)</td>
<td>pWWW0 located ILEFH1; Sm(^{\text{r}}); TOL, Km(^{\text{r}}); trp−</td>
<td>This study</td>
</tr>
<tr>
<td><em>P. putida</em> PaW340 (pWWW0::KmR::ILEFH4)</td>
<td>pWWW0 located ILEFH4; Sm(^{\text{r}}); TOL, Km(^{\text{r}}); trp−</td>
<td>This study</td>
</tr>
<tr>
<td>FH1 (pWWW0::KmR::ILEFH1)</td>
<td>pWWW0 located ILEFH1; Sm(^{\text{r}}); TOL, Km(^{\text{r}}); trp−</td>
<td>This study</td>
</tr>
<tr>
<td>FH2 (pWWW0::KmR::ILEFH2)</td>
<td>pWWW0 located ILEFH2; Sm(^{\text{r}}); TOL, Km(^{\text{r}}); trp−</td>
<td>This study</td>
</tr>
<tr>
<td>FH3 (pWWW0::KmR::ILEFH3)</td>
<td>pWWW0 located ILEFH3; Sm(^{\text{r}}); TOL, Km(^{\text{r}}); trp−</td>
<td>This study</td>
</tr>
<tr>
<td>FH4 (pWWW0::KmR::ILEFH4)</td>
<td>pWWW0 located ILEFH4; Sm(^{\text{r}}); TOL, Km(^{\text{r}}); trp−</td>
<td>This study</td>
</tr>
<tr>
<td>FH5 (pWWW0::KmR::ILEFH5)</td>
<td>pWWW0 located ILEFH5; Sm(^{\text{r}}); TOL, Km(^{\text{r}}); trp−</td>
<td>This study</td>
</tr>
<tr>
<td>FH6 (pWWW0::KmR::ILEFH6)</td>
<td>pWWW0 located ILEFH6; Sm(^{\text{r}}); TOL, Km(^{\text{r}}); trp−</td>
<td>This study</td>
</tr>
</tbody>
</table>

Km, kanamycin; \(^{\text{r}}\), resistant; \(^{\text{s}}\), sensitive; Sm, streptomycin.

### Table 2. Predicted ORFs on FH1 integron-like element in relation to plasmid pGRT1 in *P. putida* DOT-T1E.

<table>
<thead>
<tr>
<th>ORF</th>
<th>Name</th>
<th>Protein length (aa)</th>
<th>Direction</th>
<th>Amino acid (aa) identity to ORFs on pGRT1*</th>
<th>Predicted protein function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>xerD</td>
<td>385</td>
<td>←</td>
<td>ORF26; 99% in 385 aa</td>
<td>XerD-like phage integrase</td>
</tr>
<tr>
<td>2</td>
<td>int/rec</td>
<td>525</td>
<td>←</td>
<td>ORF27; 99% in 525 aa</td>
<td>Hypothetical protein with INT_REC_C conserved domain</td>
</tr>
<tr>
<td>3</td>
<td>int/rec</td>
<td>535</td>
<td>←</td>
<td>ORF30; 99% in 452 aa</td>
<td>Site-specific recombinase/phage integrase family protein with INT_REC_C conserved domain</td>
</tr>
<tr>
<td>4</td>
<td>tetR</td>
<td>138</td>
<td>←</td>
<td>ORF31; 99% in 138 aa</td>
<td>TetR family transcriptional regulator-like protein</td>
</tr>
<tr>
<td>5</td>
<td>sdiA</td>
<td>320</td>
<td>→</td>
<td>ORF32; 96% in 320 aa</td>
<td>SdiA-regulated motif containing protein on plasmid pGRT1 shown to be a modulator of the TtgGHI efflux pump in host <em>P. putida</em> DOT-T1E</td>
</tr>
<tr>
<td>6</td>
<td>dksA</td>
<td>117</td>
<td>→</td>
<td>ORF33; 98% in 117 aa</td>
<td>Hypothetical protein, DnaK suppressor-like (signal transduction mechanisms)</td>
</tr>
<tr>
<td>7</td>
<td>uspA</td>
<td>283</td>
<td>→</td>
<td>ORF34; 96% in 283 aa</td>
<td>UspA protein (universal stress response protein) on plasmid pGRT1 shown to be involved in UV response and after mild induction to increase tolerance to toluene in <em>P. putida</em> DOT-T1E</td>
</tr>
<tr>
<td>8</td>
<td>sulP</td>
<td>495</td>
<td>→</td>
<td>ORF35; 99% in 495 aa</td>
<td>Sulphate permease with STAS domain (sulphate transporter and anti-sigma factor) to be involved in siderophore production in <em>P. putida</em> DOT-T1E</td>
</tr>
</tbody>
</table>

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The FH1 ILE is diverse and associated with UV-resistance gene \textit{rulB}

The distribution of ILEs in the environment was assessed in naturally occurring pseudomonads recovered from Copper Mines Valley in the English Lake District (Cumbria, UK). From hundreds of colony-forming units (CFU) initially isolated on \textit{Pseudomonas} selective agar, 800 presumptive pseudomonad isolates were purified. Isolates were not characterized further and, because of the isolation media used, are not guaranteed to be independent isolates. Purified isolates were screened for similar ILEs by colony hybridization using the entire pFBA1001 10 kb \textit{PstI} restriction fragment as a DNA probe. This resulted in 11 positive signals (1.4%; not shown). Conjugation of hybridization positive strains with \textit{P. putida} PaW340 (pWW0) resulted in the insertion of regions of approximately 9–16 kb in size into pWW0 in 5 of the 11 isolates. In each case, the frequency of plasmid transfer ranged between $10^{-4}$ and $10^{-2}$ per recipient. Restriction fragment length polymorphism (RFLP) profiling showed that all altered pWW0 plasmids were different, and it was therefore assumed that all six ILEs were different (Fig. 1). The original bacterial isolates containing these ILEs were designated strains FH1–FH6 (Table 1), and the altered pWW0 plasmids that arose after mating with \textit{P. putida} PaW340 (pWW0) were named pWW0::ILEFH1–6.

Restriction mapping of plasmids pWW0FH2–6 using the published sequence of pWW0 as a reference (Greathed et al., 2002) suggested that as for pWW0FH1, insertion of the ILE in each case was also most likely into the \textit{rulAB} operon. Based upon the position of ILEFH1 (from pWW0::ILEFH1), insertion into pWW0 at this point would result in an unaltered \textit{rulA} gene, but with an interruption 123 bp into the \textit{rulB} gene (herein referred to as \textit{rulB}'). However, interruption at this point created an alternative ORF [\textit{rulB(2)}] encoding a predicted protein of 345 aa with a start codon at original position 6440 (Fig. 2). Fine mapping and sequencing of the region in pWW0::ILEFH1 revealed the insertion of ILEFH1 into pWW0 occurred between positions 6488–6490 in the \textit{ruvB} (\textit{rulB}) gene generating a target repeat of 5′-GAT-3′ at the insertion site (Fig. 2).

**Specificity of ILE insertion into pWW0**

The specificity of the integration was investigated by assessing insertion sites in plasmids pWW0::ILEFH1–6 by polymerase chain reaction (PCR) amplification using the primers described in Table 3. DNA from plasmids pWW0::ILEFH1–6 and the genomes of original strains FH1–FH6 and \textit{P. putida} PaW340 (pWW0) was extracted, and

![Fig. 1. RFLP profiles of \textit{HindIII} digested pWW0 plasmid variants from strains FH1–FH6. Lanes 1–6 = pWW0FH1–FH6. Lane 7 = pWW0. The size of fragments generated from \textit{in silico} digestion of pWW0 are shown for comparison.](image-url)

![Fig. 2. Insertion point and orientation of ILEs in pWW0. ILEs (orange) inserted into the \textit{rulB} gene (blue) at position 6488 on pWW0, generating the truncated \textit{rulB}' and a new predicted ORF \textit{rulB(2)}. The direct repeat created by insertion is illustrated.](image-url)
amplification was performed (Table 4). Amplification of the intact \textit{rulAB} region was successful from pWW0 DNA but not from FH1–6 genomic DNA or plasmids pWW0::ILEFH1–6. This confirmed that an intact pWW0-like \textit{rulAB} was not carried in the genomes or in pWW0 transconjugants. Amplification of the region spanning the \textit{rulAB-xerD} (590 bp) was positive for plasmids pWW0::ILEFH1–6 but negative for the genomes of original isolates and \textit{P. putida} PaW340. This indicated that in each case, the \textit{rulAB} operon had been interrupted by insertion and that a region found in ORF1 (\textit{xerD}) on the integrating region was common to all transconjugants. This was confirmed with the amplification of a region of the \textit{xerD} gene from plasmids pWW0::ILE FH1–6. These findings also showed that the six ILEs had interrupted \textit{rulAB} in the same orientation (see Fig. 2). However, at the right-hand end of the ILEs, there was variability as primers that spanned the intergenic \textit{rulB}-\textit{sulP} junction amplified from plasmids pWW0::ILEFH1 and pWW0::ILEFH5 only.

PCR products obtained from the \textit{rulAB-xerD} and \textit{rulB-sulP} primer pair amplifications were sequenced resulting in sequences for each end of the region inserted into \textit{pWW0}. In each case, it was demonstrated that insertion occurred at exactly the same position on \textit{pWW0} and generated a 5′-GAT-3′ direct repeat at the insertion point (Fig. 2).

The importance of this insertion site to the movement and integration of ILEs was tested by conjugation between strains FH1, FH4 and FH5, and \textit{P. putida} PaW340 (pWW0\_\textbackslash{\textbackslash\textbackslash}rulAB::Km}). From each of these matings, 20 transconjugants were screened for insertion into \textit{pWW0} by carrying out the \textit{xerD} PCR on extracted plasmids (because the more specific \textit{rulAB-xerD} PCR assay could not be used due to loss of the forward primer locus). Amplification did not occur (positive control DNA amplified as expected) suggesting that integration did not take place either at this original site or elsewhere on \textit{pWW0} (not shown). In matings between FH1, FH4 and FH5, and \textit{P. putida} PaW340 with the intact \textit{rulAB} carrying plasmid (pWW0::Km), this frequency of integration of ILEs was between 20% and 85% (not shown).

The sequence and location of the ILEs in the genomes of FH1, FH4 and FH5

The sequence of the ILE on pWW0::ILEFH1 ascertained from pFBA1001 elucidated the structure and location on \textit{pWW0} but did not confirm its location or structure in the genome of strain FH1. To better understand this, we obtained the draft genome sequences of strains FH1, FH4 and FH5, which based on RFLP profile, data represented three different ILEs. The ILEs within strains FH1, FH4 and FH5 were located in the draft sequences by alignment using the ILE sequences inserted into \textit{rulB} on \textit{pWW0} in each strain. Interestingly, in the case of all three strains, ILEs were located inside a chromosomal \textit{rulB} gene within a disrupted \textit{rulAB}-like operon that differed to \textit{rulAB} on \textit{pWW0} (see Fig. 3).

It was as shown that the DNA sequence of ILEFH5 shared 97% nucleotide identity with that of ILEFH1, was also 9403 bp in length and contained ORFs 1–8 that shared at least 93% protein sequence identity with those of ILEFH1 (Fig. 3).

In contrast, the ILEFH4 differed in that it was 16233 bp in length and carried 16 predicted ORFs (Fig. 3 and Table 5). The first four ORFs encoded predicted proteins...
Integron-like elements insert into rulB on plasmid pWW0

Effect of insertion of ILEs into pWW0 on UV tolerance

The effect of ILE insertion into pWW0 on host strain tolerance to UV was assessed in {P. putida} PaW340 hosts. In three independent experiments, the growth of strains {P. putida} PaW340 (pWW0::KmR), and {P. putida} PaW340 (pWW0::KmR::ILEFH1) and {P. putida} PaW340 (pWW0::KmR::ILEFH4) showed a 3 log reduction in growth after 30 s of exposure to UV (302 nm) compared with controls not exposed to UV (Supporting information Fig. S2). Plasmid-free PaW340 and PaW340 (pWW0::rulAB::KmR) both suffered 5 log reductions in CFU numbers after the same UV exposure (Supporting information Fig. S2). This suggested that insertion into rulB on pWW0 had no adverse effect on UV tolerance.

ILEs associated with rulB-like genes are present in plant and animal pathogens, and encode known virulence and fitness factors

As ILEFH1 was shown to contain similar ORFs associated with fitness-conferring traits on pGRT1, we determined whether these ILEs have a wider significance by screening the genomes of other bacteria deposited in databases for their presence. Noteworthy was the homology and structural similarities that ILEFH4 shared with regions in the 75 kb {P. aeruginosa} PA7 genomic island RGP63 (Roy et al., 2010) and the 99 kb {P. aeruginosa} genomic island PAGI-5 (Battle et al., 2008). In each of these cases, the general structure of a truncated rulAB’ operon flanking int/rec genes and tetR followed by mer genes was observed (Fig. 3). A similar structure, but lacking the tetR gene, was observed in the 123 kb {P. aeruginosa} plasmid pUM505 (Ramírez-Díaz et al., 2011). In pUM505, the overall structure differed due to interruption of the mer operon by a trpa gene (Ramírez-Díaz et al., 2011). In the genomic island RGP63, the ILEFH4-like structure was located in a region spanning 10 kb between ORF88 (designated umuC) and ORF99 (designated rvuB). This 10 kb region has been shown previously to share homology with a 9.8 kb region in genomic island PAGI-5 (Roy et al., 2010). Further analysis of this relationship in the present study has shown that the homology in this region between RGP63 and PAGI-5 is 99% over a 9.9 kb region and that in PAGI-5 the region is also bound by flanking rulB-like sequences. Significantly, on PAGI-5, this 9.9 kb is located in NR-II that has been shown to contribute to the highly virulent phenotype of host strain {P. aeruginosa} PSE9 (Battle et al., 2008).

Comparison of the sequences of ILEFH1, ILEFH4 and ILEFH5 with proposed ILEs in pGRT1, PAGI-5, RGP63,
Table 5. Predicted ORFs on the FH4 integron-like element.

<table>
<thead>
<tr>
<th>ORF Name</th>
<th>Protein length (aa)</th>
<th>Direction</th>
<th>Amino acid (aa) identity to informative database match (accession number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>xerD</td>
<td>385</td>
<td>→</td>
<td>99% in 385 aa; ORF26 in plasmid pGRT1, XerD-like phage integrase (HM626202).</td>
</tr>
<tr>
<td>int/rec</td>
<td>525</td>
<td>→</td>
<td>99% in 525 aa; ORF27 in plasmid pGRT1, hypothetical protein with INT_REC_C conserved domain (HM626202).</td>
</tr>
<tr>
<td>int/rec</td>
<td>535</td>
<td>→</td>
<td>99% in 452 aa; ORF30 in plasmid pGRT1, site-specific recombinase/phage integrase family protein with INT_REC_C conserved domain (HM626202).</td>
</tr>
<tr>
<td>tetR</td>
<td>138</td>
<td>→</td>
<td>99% in 138 aa; ORF31 in plasmid pGRT1, TetR family transcriptional regulator-like protein (HM626202).</td>
</tr>
<tr>
<td>PRDX</td>
<td>360</td>
<td>→</td>
<td>89% in 360 aa; peroxiredoxin in Pseudomonas sp. GM49 (ZP_10658778).</td>
</tr>
<tr>
<td>HP</td>
<td>229</td>
<td>←</td>
<td>90% in 41 aa; hypothetical protein with sequence similarity to a region of Tn5041 in Pseudomonas sp. (CAC80074).</td>
</tr>
<tr>
<td>merR</td>
<td>139</td>
<td>←</td>
<td>97% in 139 aa; putative transcriptional regulator MerR in P. aeruginosa (NCGM1179).</td>
</tr>
<tr>
<td>merT</td>
<td>134</td>
<td>←</td>
<td>78% in 104 aa; mercuric transport protein MerT in P. aeruginosa PA7 (ABR82023).</td>
</tr>
<tr>
<td>merP</td>
<td>134</td>
<td>←</td>
<td>99% in 91 aa; putative MerP protein component of transporter in P. mandelli JR-1 (ZP_11114267).</td>
</tr>
<tr>
<td>merC</td>
<td>144</td>
<td>→</td>
<td>90% in 143 aa; putative MerC superfamily protein in P. mandelli JR-1 (ZP_11114268) and P. aeruginosa ATCC 700888 (ZP_15625973).</td>
</tr>
<tr>
<td>merA</td>
<td>581</td>
<td>→</td>
<td>95% in 560 aa; mercuric reductase protein MerA in P. mandelli JR-1 (ZP_11114269).</td>
</tr>
<tr>
<td>merD</td>
<td>120</td>
<td>→</td>
<td>100% in 120 aa; mercuric resistance transcriptional repressor MerD, MerR family in P. mandelli JR-1 (ZP_11114271).</td>
</tr>
<tr>
<td>merE</td>
<td>79</td>
<td>→</td>
<td>96% in 77 aa; MerE superfamily mercury resistance protein in P. mandelli JR-1 (ZP_11114272).</td>
</tr>
<tr>
<td>terC</td>
<td>515</td>
<td>←</td>
<td>96% in 515 aa; TerC superfamily integral membrane protein in Pseudomonas sp. UW4 (YP_007029200).</td>
</tr>
<tr>
<td>rulB-like</td>
<td>160</td>
<td>←</td>
<td>60% in 104 aa; putative ImpB/MucB/SabB/RulB family protein of DUF4113 superfamily in P. stutzeri TS44 (ZP_1447253).</td>
</tr>
</tbody>
</table>

pUM505 and another candidate region on the chromosome of P. syringae pv. tomato DC3000 showed that all share structural features with the recently proposed type III protein secretion system effector (T3SE) ILEs (Jackson et al., 2011). T3SE ILEs have T3SE gene(s) orientated so that the transcription is towards the 3’ end of the integrase gene and therefore not under the influence of the integrase P$_r$ promoter. Although we have not identified T3SE genes on the ILEs here, this feature is shared with the integrated genes downstream of tetR in the ILE$_{FH1}$ and ILE$_{FH5}$, and on pGRT1, but not with all sequences down-stream of tetR in FH4, pUM505, PAG1-5 and RGP63 (Fig. 3). In addition, we have been unable to demonstrate the presence of a P$_r$ promoter in the upstream integrase gene. However, even if present, its influence would not be exerted on rulA or disrupted rulB’ that flank the element since they are transcribed in the opposite direction. In T3SE ILEs, insertion into the rulAB operon is considered likely to be under the influence of the LexA repressor because of a LexA binding region in the rulAB promoter (Jackson et al., 2011). Consistent with this, we found LexA1 binding sites with the characteristic CTG-N$_{10}$-CAG motif upstream of rulA in each of the chromosomally located ILEs of FH1, FH4 and FH5 as well as plasmids pWW0, pGRT1 and genomic islands PAG1-5, RGP63 and pUM505 (Fig. 4A).

In T3SE-integrons, it was also observed that the integrase gene was situated less than 100 nucleotides downstream of the 5’ end of the truncated rulB’ gene, and each case lacked its own upstream LexA or RpoD binding site (Jackson et al., 2011). In P. syringae pv. tomato DC3000 plasmid A and P. syringae pv. pisi avrPpiA chromosome site, both of which were described as carrying ‘complete’ T3SE ILEs, the integrase gene was 60 bp from the end of rulB (Jackson et al., 2011). More significantly, in the present study, we observed that the start codon of the xerD integrase gene was either 118 nt (ILE$_{FH1}$) or 119 nt (ILE$_{FH1}$ and ILE$_{FH5}$) from the GAT point of insertion at the end of truncated rulB (rulB’; Fig. 4B). For the avrPpiA-containing element and that on DC3000 plasmid A, both of which contained a predicted rulB’ ORF, this GAT triad is also found 118 bp upstream of the integrase start codon ATG (Fig. 4B). This was also the case for the putative ILEs in PAG1-5, RGP63 and plasmid pUM505 (Fig. 4B). In pGRT1, the relationship with ILE$_{FH1}$ and ILE$_{FH5}$ was strengthened with the distance also being 119 nt (Fig. 4B). While we have no evidence regarding the specific site of integration in each of these other putative ILEs, we cannot rule out the potential importance of this observation to the integration of this family of ILEs in general.

**Analysis of the ILE insertion site in different genomes**

To investigate the potential for insertion into rulB-like regions and the extent to which it may have already occurred in the genomes of other bacteria, we performed
DNA alignments using 123 bp regions that spanned 60 bp on either side of the insertion site of both the intact and interrupted pWW0 rulB gene (Supporting information Fig. S3). The intact region of pWW0-rulB aligned with five sequences originating in catabolic plasmids (pND6-2, pDTG1, pNAH7, pNAH20 and KOPRI126573) from *Pseudomonas* spp. (Supporting information Fig. S3A). Five sequences of different origin to those earlier were identified with homology to the two 123 bp rulB-ILE junctions, of which four aligned with both ends. As previously, these four aligning sequences were from plasmids pGRT1, pUM505 and genomic islands PAGI-5 and

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RGP63. In each case, the pWW0 insertion point was preserved at the xerD side, and the 60 bp in the intergenic region between the insertion point and xerD contained three highly conserved regions including a 7 bp sequence (CTGAGGG) immediately inside the insertion point (Supporting information Fig. S3B). However, these conserved regions were not found in the proposed ILEs in pDC3000A or in that harbouring the avrPpiA gene (Fig. 4B). At the right-hand side of the element, the 60 bp of the intergenic region was similarly conserved despite ORFs on this side being variable (Supporting information Fig. S3C). In each of the aligning DNAs the 60 bp on the outside of each of these intergenic regions was shown to be a rulB-like sequence indicative of an insertion event having already taken place.

Despite the sequence conservation at each end of the mobile regions, repeat regions that might be involved in movement of the element were not found, and the significance of each of these conserved regions is not presently understood.

**Phylogenetic analysis of the ILEs and their host strains**

Strains FH1–FH6 were identified as *Pseudomonas fluorescens* by API20E biochemical tests (not shown). Alignment of 797 bp of the gyrB gene obtained from the draft genomes of FH1, FH4 and FH5 with their closest relatives is shown in Supporting information Fig. S4. All three strains were placed within the *P. fluorescens* species complex, with FH1 and FH5 being located in the *P. fluorescens* subgroup with closest relatives being *P. extremorientalis* LMG 1965T (FH1) and *P. libaniensis* CIP 105460T (FH5). Strain FH4 was placed within the *P. gessardi* subgroup with *P. brenneri* DSM 15294T as its closest relative (Mulet et al., 2010). This phylogenetic grouping corresponded well to the relationship of the ILEs characterized here, whereby ILEFH1 and ILEFH5 were very closely related but different to ILEFH4. Further analysis of the three phage integrase/site-specific recombinase genes and comparison with those of other ILEs confirmed this (Supporting information Fig. S5) and suggested that ILEs may have been associated with different clades of *P. fluorescens* group bacteria for some time.

**Discussion**

A key objective in understanding bacterial evolution is to gain insight to the various mechanisms underpinning genotypic and phenotypic changes. By examining the outcome of plasmid conjugation events between environmental *Pseudomonas* bacteria, we have discovered a new set of genetic elements, reporting for the first time the observation of active site-specific integration of a novel and related group of ILEs into the rulAB operon on plasmid pWW0. The environmental pseudomonads described here were isolated between 18 and 28 years ago, and from a relatively small sample of cultured pseudomonads. The frequency of confirmed ILEs within this sample group (n = 800) was 0.75%, which suggests that the number of this family of ILEs alone in the environment is likely to be large and of significance to the transfer of fitness or virulence/avirulence traits between bacteria. Based on DNA and protein homology and similar structural features, we have proposed that other members of this group exist in genomes and plasmids integrated into rulB-like genes.

The site-specific insertion of ILEs carrying adaptive traits into the rulB locus is key to the overall significance of this study as it signifies a potential hotspot for integration of what appear to be atypical integrons that are not primarily associated with acquisition and carriage of antibiotic resistance cassettes (see Cambray et al., 2010). Typically, integrons are gene capture systems that comprise a core stable platform of an intI gene (a tyrosine recombinase) with its own promoter (Pint), and an outward facing promoter (Pc) that can express captured cassettes, and an adjacent upstream attR recombination site (Cambray et al., 2010) into which cassettes are captured by recombination with the cassette attL site. The ILEs described here differ to this typical structure. First, the intI-like gene (ORF1; xerD) does not appear to contain promoters Pint or Pc, and even if they were present, the gene is oriented in the opposite direction to typical integrons so that Pc would have no effect on expression of the genes in the ‘variable side’ of the ILE. Second, the orientation of xerD in ILEs suggests that the attR site would be in the region where integration into rulB occurs. However, we could not find any such attR recombination site adjacent to xerD or elsewhere in these ILEs.

ILEs described here are of two types based on the small sequence differences in the left-hand ‘conserved side’ and different ORFs present in the right-hand ‘variable side’. This variation also appears to reflect the bacterial lineages from which they were derived. ILEFH1 and ILEFH5 share closest homology with each other, and both originated in host bacteria within the *P. fluorescens* subgroup, while ILEFH4 had a different variable side and originated in a *P. gessardi* subgroup host. In ILEFH1–FH5, the variation in ORFs carried downstream ORFs 1–3 (the three recombinase family ORFs) was akin to the variation in cassettes carried by typical integrons (see Cambray et al., 2010). ORFs downstream of the recombinases in ILEFH1 and ILEFH5 (ORFs 1–3) shared > 96% homology with counterparts on plasmid pGRT1, whereas ORFs in ILEFH4 shared homology with those on *P. aeruginosa* genomic islands PAGI-5 and RGP63, and plasmid pUM505. We have not determined the effects on host fitness resulting from insertion of ILEs into rulB on pWW0.
Integron-like elements insert into rulB on plasmid pWW0

Beyond UV tolerance assessments as a more encompassing assessment of the wider environmental distribution and traits conferred by ILEs is planned. However, based on the evidence in the literature, it is likely that traits conferred by ILEs are of major significance to plant and animal health. In their report on plasmid pGRT1, Molina and colleagues (2011), assessed traits conferred by several of the ORFs located between ORFs 25 and 36 (the region nearly identical to the ILEFH1), and showed that some conferred a selective advantage on the host bacterium including the modulation of toluene efflux pump genes located on the chromosome of the host bacterium P. putida DOT-1E (see Table 2).

In the genomic island PAGI-5, the region that shared homology with ILEFH resided within NR-II, which has been shown to make a substantial contribution to the virulence of the host bacterium P. aeruginosa PSE9 (Battle et al., 2008). In PAGI-5, NR-II spans ORFs 40–62 (approximately 17.5 kb) of which ORFs 49–60 share homology and structural similarities with ILEFH ORFs 1–11. It is unknown whether the whole 17.5 kb NR-II sequence is required for virulence or whether it is due to a smaller region such as ORFs 49–62 or the ORFs of unknown function (encoding hypothetical proteins) (ORFs 40–48). However, the independent movement and integration of a region with close homology to a key virulence region in animals is extremely significant. This is particularly pertinent when it is considered that similar regions to NR-II were present in six other P. aeruginosa PSE strains (PSE11, 15, 17, 30, 35 and 39) (Battle et al., 2008).

While in the present study, interruption of rulB by ILEs in pWW0 was observed in laboratory experiments only, there is evidence that an almost identical rulB (rulB) gene on an IncP-9 pWW0-like plasmid, pDTG1, has previously served as an insertion hotspot in the natural environment. Plasmid pDTG1 contains a disrupted rulB gene and shares considerable structural and sequence similarity with pWW0, and both are thought to have had a common predecessor (Dennis and Zylstra, 2004). In pDTG1, the rulB gene has been disrupted by insertion of a 6 kb region thought to be derived from plasmid pCAR1 and prior to further insertion of genes encoding naphthalene degradation (Dennis and Zylstra, 2004). From sequence analysis of the present newly discovered ILEs and of genomes deposited in databases, we have found no evidence of interruption of the rulA gene (or rulA-like genes) by insertion. However, rulB, or its homologous gene in other bacteria, is frequently seen to be disrupted in other bacterial genomes.

The rulAB operon (either intact or interrupted) is often situated close to integrase genes and other fitness/effector/virulence genes in the genomes of pseudomonads. This association extends to rulAB relatives such as rumAB, mucAB, umuDC and samAB in other genera (see Böltnner et al., 2002; Dennis and Zylstra, 2004; Li et al., 2004; Stavrinides and Gutman, 2004; Sundin et al., 2004; Zhao et al., 2005; Wozniak et al., 2009; Wozniak and Waldor, 2010; Seth-Smith et al., 2012). In several of these cases, a rulB-like gene (umuC, mucB, impB and rumB) is interrupted by a region containing an inte格rase family gene. Perhaps most noteworthy of these is the SXT-R391 family of integrative and conjugative elements (ICEs) that share 52 core genes as well as five intergenic hotspots for insertion (known as HS1-HSS; see Wozniak et al., 2009). Outside of these hotspots are other regions that contain variable DNA. In the cases of the element SXT and the ISCR2-like elements, ICEpdaSpa1, ICEPalBan1, ICEVchInd5, ICEVchBan5, ICEVchBan9/ICEVchMoz10 and ICEVflInd1, the variable regions are inserted into rumB (Wozniak et al., 2009). None of these elements have relationships with those described here other than that significantly, they reiterate a feature of the umuC-encoding subfamily locus in being a hotspot for the insertion of mobile DNAs.

Possible reasons as to why insertion of these ILEs is specific to the rulB gene in this case and possibly widespread in nature in rulB-like homologues remain unclear. Proteins RulA and RulB are members of the UmuC-like subfamily of lesion-replicating Y-family DNA polymerases (alongside UmuDC, MucAB, ImpAB and RumAB) that are encoded in the chromosomes and plasmids of numerous bacteria. In Pseudomonas spp., the role of the rulAB operon in the SOS response and the general adaptational traits of the host (Tark et al., 2005; Sundin and Weigand, 2007) would suggest that disruption of rulB by an insertion event might be detrimental to the host. However, if this interruption did not significantly alter the functionality of RulA or RulB, or the traits acquired by insertion provided a greater fitness benefit than encoded by an intact rulAB operon alone, then perhaps selection would be favoured. Interruption of rulB at position 6488 on pWW0, as occurred in the present study, did not result in a reduction in UV tolerance (Supporting information Fig. S2). This may suggest that ORF rulB(2) encodes a functional protein RulB(2) similar in function to the original RulB (see Fig. 3).

It appears that insertion into rulB guarantees some measure of vertical mobility (from chromosome to plasmid within the same host), and this may be extended to horizontal mobility, as more often than not in plasmids (including pWW0), the rulAB operon is found close by replication and transfer functions (Gibbon et al., 1999).

The presence of conserved features in the left-hand side of the ILEs such as an interrupted rulB, a downstream conserved 118–119 bp intergenic region and a conserved xerD-like inte格rase/recombinase followed by two other site-specific recombinase genes may be indicative of a minimum requirement for this integration and
resolution. As these ILEs can move from an interrupted chromosomally located rulB-like gene into another, it suggests that the rulB gene may form part of this minimum region and that homologous recombination may be involved. However, to date, we have been unable to locate regions sequences at the ends or within ILEs that might be evidence of the usual means of insertion such as homologous recombination, transposition and site-specific recombination.

It is important for future studies to determine the mechanisms and driving force behind this movement of ILEs into pWW0 and possibly other loci. We are presently investigating the mechanisms for the movement of ILEs based on evidence that antibiotics (Guerin et al., 2009; Guerin et al., 2011) and mechanisms of horizontal gene transfer such as conjugation and transformation may trigger the integration of ILEs into rulB through induction of the integron integrase (Bahraloglu et al., 2010; 2012; Cambray et al., 2011).

Concluding remarks

The demonstration here of the active and repeatable integration of related fitness-gene carrying ILEs into rulB on pWW0 and the presence of intact rulAB (and other UmuC subfamily protein encoding genes) on plasmids and chromosomes suggests that there exists a candidate region in bacteria that can be used to monitor the acquisition and movement of fitness-conferring traits. Additionally, this region might offer a means of capture of novel ecologically and perhaps clinically significant fitness-related elements and allow an understanding of potential virulence, avirulence and fitness-related traits that could impact on plant and animal health. An excellent example of a candidate group with which to test this idea are the pPT23A family plasmids (PFPs) (see Ma et al., 2007). This large family contains plasmids harbouring a range of fitness-related genes. In a study of 31 plasmids from this family in pathovars of P. syringae (Zhao et al., 2005), the full sequence of six PFP plasmids and microarray analysis of 161 genes from the remaining 25 showed that 19 of the 31 contained both rulA and rulB, and that a further seven contained rulB alone (Zhao et al., 2005). This study of plasmids from this family and other sources will form the basis of future studies.

Experimental procedures

Bacterial strains, plasmids and sampling

Bacterial strains and plasmids are described in Table 1. Escherichia coli strains and P. putida PaW340 were maintained on nutrient agar (NA, Oxoid, Basingstoke, UK). Antibiotics used in media were either made up fresh on the day of use or stored at −20°C as 1000× concentration stock solutions.

Environmental isolate FH1 was recovered in 1985 from a laboratory facility in the grounds of the Freshwater Biological Association (Far Sawrey, Cumbria) that received freshwater from Windermere in the English Lake District. Environmental pseudomonads were recovered from sediment/water samples collected in sterile 500 ml bottles in 1995 from Deep Adit, a horizontal drainage shaft that flows into Red Dell Beck from the disused copper mine in Copper Mines Valley (Coniston, Cumbria, UK; National Grid Reference SD290987) (Pickup, 1989). Samples were stored at 4°C for up to 2 days before processing. Pseudomonads were isolated on Pseudomonas selective agar (Oxoid, UK) 20°C for up to 5 days, and were purified and maintained on nutrient agar.

Identification of isolates

All ILE-containing isolates were initially confirmed within the genus Pseudomonas by using API 20 NE test strips (Biomerieux, Basingstoke, UK). Deeper phylogenetic placement of selected isolates was carried out based on alignment the gyrB gene (Mulet et al., 2010) obtained from genome sequencing (see later discussion).

Conjugation experiments

Filter matings were performed by separately resuspending a loop full of freshly cultured donor and recipient cells in 300 μl 1× phosphate buffered saline (PBS) (pH 7.4) followed by overlaying 10 μl of each suspension on to a 0.22 μm pore
size membrane filter (Sупор-200, Pall Life Sciences, Portsmouth, UK) on nutrient agar medium and incubation at 28°C (± 0.5°C) for 24 h. Controls (unmixed donors and recipient cells) were treated in the same manner. After incubation, cells and controls were resuspended in 450 μl PBS and transconjugants were selected by spreading onto M9 agar supplemented with the required amino acids and antibiotics to select for transconjugants and against donors and recipients (see Table 1). All transconjugants were confirmed by conferring the required plasmid phenotype in addition to resistance or sensitivity to streptomycin and the requirement for the addition of tryptophan to M9 minimal medium.

Plasmid transfer frequency was determined by growth on M9 medium supplemented with glucose (10 mM) and kanamycin (25 μg ml⁻¹), and without the addition of tryptophan (to select against PaW340). Briefly, donor and recipient were cultured in nutrient broth (NB) with antibiotics as required followed by serial dilution in sterile PBS. From these dilutions, spread plating was carried out on non-selective nutrient agar (NA) to determine cell concentrations of donor and recipient. Serially diluted donor and recipient cultures were also mixed (50 μl of each) and spread plated on to selective M9 agar as earlier. Transfer frequency of pWW0 was expressed as transconjugants per recipient cell. Twenty transconjugants from each mating were screened by PCR for the presence of the inserted element using the rulAB-xerDFP and rulAB-xerDRP primer set (see Table 3), and the transfer was expressed as integrations per transconjugant.

ILE insertion specificity

ILE insertion specificity into rulB on pWW0 was investigated by filter matings between strains FH1, FH4 and FH5, and PaW85 (trpΔ::KmR). Plasmid transfer frequency was determined by growth on M9 medium supplemented with glucose (10 mM) and kanamycin (25 μg ml⁻¹), and without the addition of tryptophan (to select against PaW340). Briefly, donor and receiver were cultured in nutrient broth (NB) with antibiotics as required followed by serial dilution in sterile PBS. From these dilutions, spread plating was carried out on non-selective nutrient agar (NA) to determine cell concentrations of donor and recipient. Serially diluted donor and recipient cultures were also mixed (50 μl of each) and spread plated on to selective M9 agar as earlier. Transfer frequency of pWW0 was expressed as transconjugants per recipient cell. Twenty transconjugants from each mating were screened by PCR for the presence of the inserted element using the rulAB-xerDFP and rulAB-xerDRP primer set (see Table 3), and the transfer was expressed as integrations per transconjugant.

IEL insertion frequency

The frequency of ILE integration into rulB was assessed by PCR amplification of the rulB-xerD (Table 3) region in 20 confirmed transconjugants after cell lysis at 95°C in sterile PBS. Cell lysis was confirmed in each case by incorporation of the xerD region from transconjugants. Frequency of integration was expressed as percentage of rulB-xerD positives to xerD positives.

UV tolerance assessments

UV tolerance experiments were carried out using a similar method to that of Molina and colleagues (2011). The strains PaW85 (trpΔ::KmR) and PaW340 were inoculated into isosensitest agar plates and directly exposed to UV at a distance of 1 cm at 15 s intervals up to 1 min. Control plates were not exposed to UV. Twenty independent assays were carried out with duplicate plates in each.

Plasmid extraction

Plasmid DNA was extracted from control strains and transconjugants after growth in the required selective media at 30°C with shaking at 150 r.p.m. for 18 h using QIAGEN mini and midi columns (Qiagen, UK).

PCR amplifications

PCR amplifications were carried out in individual thin-walled 0.2 ml tubes on a Veriti thermal cycler (Life Technologies, Paisley, UK). PCR primers were designed using the Primer 3 software (http://primer3.wi.mit.edu/) (Untergasser et al., 2012) (Table 3). Amplified DNA was visualized by agarose gel electrophoresis in gels stained with ethidium bromide and excised from the gel using the QIAGEN gel extraction kit II (Qiagen, UK).

DNA sequencing, annotation and analysis

PCR products were purified using QIAquick PCR purification kit (Qiagen, UK) and sequenced on the top strand directly from the forward primer of the reaction using QIAGEN genomic services (Qiagen, Düsseldorf, Germany).

The 10 kb region of pWW0::ILEFH1 in pFBA1001 was sequenced commercially (Qiagen, Germany) by Dye Terminator cycle sequencing (using a Model 3730XL automated DNA Analyser; Life Technologies) of pUC19-based shotgun clones to at least six times coverage and accuracy assured to at least 99.995%.

The draft genomes of strains FH1, FH4 and FH5 were sequenced using the Illumina HiSeq platform (Illumina, Cambridgeshire, UK). De novo assembly was performed using Velvet with settings selected using VelvetOptimiser (http://www.vicbioinformatics.com/software.velvetoptimiser.shtml). DNA (BLASTn) and protein (BLASTp) alignments, and ORF analysis (ORF Finder) were carried out using NCBI suite of facilities (http://www.ncbi.nlm.nih.gov). Multiple sequence alignments were performed and annotated using CLUSTALW (Thompson et al., 2002). Phylogenetic tree construction was carried out using the ‘One Click’ mode within the facilities found at http://www.phylogeny.fr (Dereeper et al., 2008; 2010). Graphical representations of DNA were performed manually or using SnapGene V1.4 software (http://www.snapgene.com).

Nucleotide sequence accession numbers

The DNA sequence of the 10.1 kb region of plasmid pFBA1001 has been deposited in DDBJ/EMBL/GenBank.
under the accession number KC81795. The Whole Genome Shotgun project data for strains FH1, FH4 and FH5 have been deposited at DDBJ/EMBL/GenBank under the accession numbers AOHM00000000, AOHN00000000 and AOJA00000000 respectively. The versions described in this paper are versions AOHM10000000, AOHN10000000 and AOJA10000000 respectively.

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mobilized class 1 integrons are common in the chromosomes of pathogenic Pseudomonas aeruginosa clinical isolates. *Antimicrob Agents Chemother* **56**: 2169–2172.


Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site: